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Coupling of early response gene expression to distinct regulatory pathways during α-interferon and phorbol ester-induced plasmacytoid differentiation of B chronic lymphocytic leukaemia cells

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Phorbol esters (phorbol 12-myristate 13-acetate; PMA) and α-interferon (α-IFN) act through divergent signal transduction pathways to induce B chronic lymphocytic leukaemia cells (B-CLL) to undergo plasmacytoid differentiation in vitro. By using a panel of PMA-inducible early response gene probes we show that these two different effectors are coupled to second messenger pathways that do not converge on a common gene regulatory programme in differentiation of B-CLL cells. Moreover, using the calcium ionophore, A23187, four distinct regulatory classes of early response gene could be defined implying that multiple regulatory pathways may mediate the process of terminal differentiation in B lymphocytes.

Early response gene regulation; Phorbol ester; B lymphocyte differentiation

1. INTRODUCTION

B-cell chronic lymphocytic leukaemia cells (B-CLL) represent a monoclonal expansion of malignant B lymphocytes arrested at a stage of differentiation intermediate between the pre-B cell and the resting mature peripheral blood B lymphocyte [1,2]. This differentiation arrest can be overcome by treatment of the cells with a variety of agents such as phorbol ester (phorbol 12-myristate 13-acetate; PMA) and various lymphokines particularly α-interferon (α-IFN) [3–6]. In vitro induced plasmacytoid differentiation of B-CLL cells provides a useful model system in which to study mechanisms that co-ordinately regulate gene expression during terminal differentiation of B lymphocytes in response to extracellular signals. Recent studies have shown that PMA-induced plasmacytoid differentiation of B-CLL cells is preceded by the initiation of a complex gene regulatory programme characterised by a rapid transient stimulation in expression of a large number (probably $>10^2$) of early response genes (ERGs) [7], which include in addition to members of the nuclear proto-oncogene families many anonymous genes of unknown function [7,8]. The effects of PMA on many cell types, including lymphocytes are thought to be largely mediated by substitution for diacylglycerol in the direct activation of the protein kinase C (PKC) signal transduction pathway [9]. In contrast, the second messenger pathways activated by α-IFN remain largely unknown but do not appear to involve PKC nor the other classical second messenger pathways coupled to intracellular calcium or cyclic adenosine monophosphate [10].

In this report we have used a panel of ERG probes to investigate the relationship between the gene regulatory pathways activated by PMA and those activated by α-IFN in B-CLL cells. Our results show that only a minor subset of PMA-responsive ERGs are induced by α-IFN indicating that the divergent signal transduction pathways coupled to these two different effectors do not converge on a common gene regulatory programme in terminal B lymphocyte differentiation. In addition, using the calcium ionophore, A23187, we show that 4 regulatory classes of ERG can be defined on the basis of expression characteristics implying that multiple gene regulatory pathways may mediate the process of induced terminal differentiation in B lymphocytes.

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Abbreviations: B-CLL, B cell chronic lymphocytic leukaemia; ERG, early response gene; PMA, phorbol 12-myristate 13-acetate; α-IFN, α-interferon

2. MATERIALS AND METHODS

2.1. *Materials*

Phorbol 12-myristate 13-acetate and A23187 were purchased from Sigma (Poole, Dorset, UK). α-IFN was obtained from Kirby Warrick Pharmaceuticals (Suffolk, UK) and [α-^{32}P]dCTP was obtained from Amersham International (Bucks, UK). Cell culture materials were obtained from Gibco (Grand Island, NY, USA).

2.2. *Cell isolation and culture*

Mononuclear cell preparations were obtained from B-CLL patients with peripheral lymphocyte counts $>60 \times 10^9$/l by a single step gra-

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dient centrifugation on Ficoll-Hypaque and depleted of monocytes by adherence to plastic. Freshly prepared B-CLL populations contained <1% monocytes and <5% T lymphocytes as judged by flow cytometry and immunofluorescence with T cell-specific monoclonal antibody (CD3) (data not shown). After overnight incubation in serum-free RPMI-1640 medium B-CLL cells were incubated (10^6 cells/ml) in RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM glutamine and 20 μg/ml gentamicin with or without previously determined optimal concentrations of PMA (30 nM), A23187 (250 nM) alone or in combination with PMA (30 nM), or α-IFN (500 units/ml). Cells were maintained at 37°C in a humidified incubator.

2.3. *RNA hybridisation analysis*

At various times B-CLL cells (10^8) were harvested and total RNA was extracted by the guanidium isothiocyanate procedure [11]. 2 μg samples per time point were electrophoresed through 1% agarose gels in 0.8 M formaldehyde [12]. RNA was blotted on nylon filters and hybridised to ^{32}P-labelled probes (10^6 cpm/ml) for 24 h. Filters were then washed with 2 × SSC, 4 times (30 s), with 2 × SSC 4 times (20 min), 1% SDS, in 2 × SSC at 60°C for 20 min and finally at 60°C in 0.1 × SSC (20 min). Autoradiography was performed at −70°C using Dupont, 'Lightening Plus' intensifying screens for 1–14 days.

The c-myc probe was a 1.5 kb *Eco*RI-*Hin*dIII genomic fragment containing the second myc exon [13]. The c-fos probe [14] was a 1.5 kb *Eco*RI cDNA fragment (J.J. Murphy and J.D. Norton, unpublished). A panel of 12 PMA-induced B-CLL ERGs previously cloned and characterised were also used in these experiments [7].

3. RESULTS

The expression characteristics of 14 ERGs were studied after stimulation of B-CLL cells with either PMA, α-IFN or the calcium ionophore, A23187. Expression was also analysed following stimulation of cells with PMA and A23187 together, since these agents have previously been shown to act synergistically on many cell types including B-CLL cells [15]. The ERG panel comprised the proto-oncogenes c-fos and c-myc, together with a further 12 'anonymous' ERG probes described previously [7].

Expression of c-myc was stimulated by PMA alone but no detectable increase in expression was observed after induction of cells with either α-IFN or A23187 (Table I, Fig. 1). A further 7 ERGs showed similar expression characteristics to c-myc (illustrated for ERG 19A in Fig. 1) with upregulation only in response to PMA but not with α-IFN or A23187.

c-myc and c-fos were not coordinately regulated. In contrast to c-myc, c-fos and another ERG, 3L11, were induced by both PMA and A23187 and to a lesser extent by α-IFN (Table I, Fig. 1). Thus all 3 activating agents induced the ERGs c-fos and 3L11 to varying amounts. ERGs 1R20 and 1R19 were induced in B-CLL cells following stimulation by both PMA and α-IFN but not by the calcium ionophore (Table I, Fig. 1). In contrast, ERGs 1L3 and 4B1 were induced by PMA and A23187 but not by α-IFN (Table I, Fig. 1). Interestingly, ERG 1L3 was the only gene which showed increased expression following stimulation with PMA and A23187 together compared to stimulation with PMA alone (Table I, Fig. 1).

Table I

Induction of early response genes by different agents relative to the PMA-induced response

Gene	A23187	PMA + A23187	α-IFN
c-myc	−	+ + +	−
c-fos	+ +	+ + +	+
1L3	+ +	+ + + +	−
1L4	−	+ + +	−
1R19	−	+ + +	+ +
1R20	−	+ + +	−
1R21	−	+ + +	+ +
3L2	−	+ + +	−
3L3	−	+ + +	−
3L11	+ +	+ + +	+ +
4B1	+ +	+ + +	−
5L3	−	+ + +	−
10A	−	+ + +	−
19A	−	+ + +	−

+ + + = level of induction of early response genes by PMA alone

ERGs induced by either PMA or calcium ionophore showed similar kinetics of expression (Fig. 1). However, α-IFN-stimulated expression of ERGs 1R19 and 3L11 was delayed compared to PMA-induced ex-

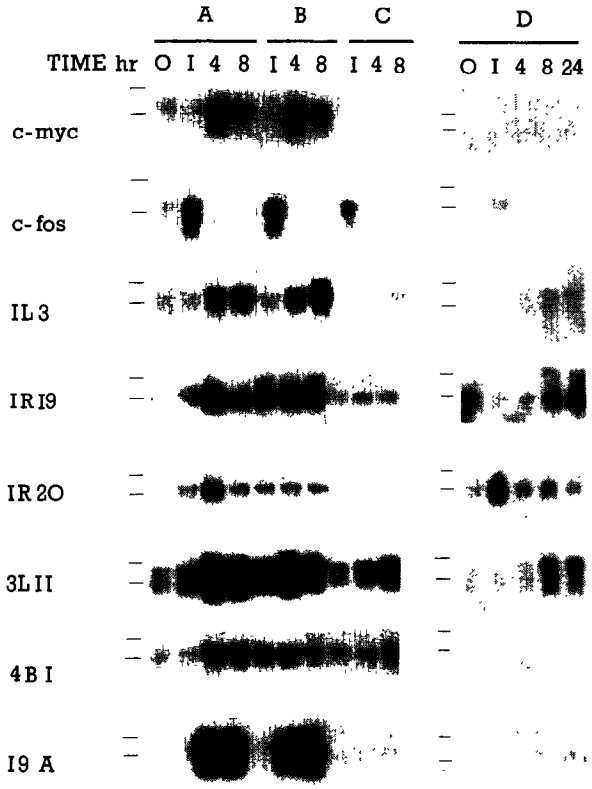

Fig. 1. Northern blot time course of ERG expression in B-CLL cells after stimulation with different agents. B-CLL cells were stimulated for the times indicated with (A) PMA (30 nM), (B) PMA (30 nM) and A23187 (250 nM) together, (C) A23187 (250 nM) alone or (D) α-IFN (500 units/ml). Total RNA was isolated and Northern blots were hybridised with gene probes. The position of the 28 S and 18 S ribosomal RNA bands are shown.

pression, and α-IFN-stimulated expression of ERG 1R20 was more rapid and transient than with PMA (Fig. 1).

4. DISCUSSION

Using B-CLL cells as a model, the relationship between the early gene regulatory programme elicited by three different activating agents; PMA, α-IFN and the calcium ionophore, A23187, was studied during in vitro induced plasmacytoid differentiation of B lymphocytes. Although the 12 'anonymous' ERGs used in the present study were previously cloned on the basis of their inducibility in response to PMA in B-CLL cells [7], a sizable proportion of them were found to be induced in response to α-IFN and/or A23187.

Calcium ionophores have been reported to act synergistically with PMA on a number of cell types including B lymphocytes to enhance the differentiative response [15]. However, Klemtz et al. previously reported no synergistic effect of A23187 on PMA-induced expression of the nuclear proto-oncogenes c-fos and c-myc in mouse B lymphocytes [16]. Consistent with this we found that, with the exception of the ERG 1L3, A23187 did not have any synergistic effect on PMA-induced expression of ERGs in B-CLL cells. This suggests that the potentiating effects of ionophores on PMA-induced plasmacytoid differentiation is mediated through a relatively limited number of ERGs.

Studies on normal B lymphocytes have also shown that calcium ionophore alone stimulates expression of both c-fos and c-myc [16]. In contrast, we found that in B-CLL cells only c-fos was induced in response to treatment with ionophore alone. These differences may well reflect changes in calcium-dependent signalling pathways associated with malignant B lymphocytes.

In contrast to calcium ionophore which by itself does not affect significant phenotypic changes in B-CLL cells [15], α-IFN induces a plasmacytoid differentiative response similar to that elicited by PMA [5,6]. However, the observation that only 4 of the 14 ERGs were induced by α-IFN rather implies that these two different effectors activate signal transduction pathways which do not converge on a common gene regulatory programme in terminal B lymphocyte differentiation. Interestingly, we recently found that PMA-induced expression of the α-IFN-responsive genes c-fos, 1R19 and 3L11 and the A23187-responsive genes 4B1 and 1L3 is mediated through a protein kinase C-independent mechanism (J.J. Murphy and J.D. Norton, submitted for publication). This raises the possibility that these ERGs are coupled to signal transduction pathways activated by both PMA and α-IFN/A23187. Alternatively, expression of these ERGs may be coupled through divergent signal transduction pathways to distinct PMA/α-IFN/A23187-responsive *cis*-regulating determinants shared by each gene. Such an explanation may well account for the expression characteristics of the c-fos gene which contains, in addition to the classical serum response element [17] and 'AP1'-like site [18] involved in PMA-responsiveness, a short region within the promoter domain displaying homology to interferon response elements (see [19]).

Whilst the precise regulatory determinants of different ERGs in B cells remain to be determined, our data reveal 4 distinct regulatory classes that can be defined on the basis of induced expression characteristics: (i) genes induced by PMA alone, (ii) genes induced by PMA and α-IFN, (iii) genes induced by PMA and calcium ionophore, (iv) genes induced by all three effectors. These observations imply that multiple gene regulatory mechanisms act on B lymphocytes during the process of induced terminal differentiation.

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